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Gamma-irradiation Induces Phenotypic and Genotypic Mutations in M3 and M4 Generations of *Phaseolus vulgaris*

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GAMMA irradiation might be an alternative strategy for increasing crop yield and/or introducing new varieties. Reddish-brown *Phaseolus vulgaris* L. cv Abo Yousuf was exposed to 50Gy of γ-radiation produced M2 seeds, while 100 and 300Gy were lethal. M2 seeds were cultivated to investigate the segregated traits in M3 and M4 plants. Segregation of purple and green: hypocotyls, lower venation, and pods, besides purple flowers and emarginated leaf apex, were observed in M3 plants. M3 seeds showed five seed coat colors: reddish-brown, brown, dark brown, grayish brown, and gray. The reddish-brown seeds had the highest weight, 30.74%, whereas the gray seeds had a much lighter weight, 23.14%. The flower color was light purple in three lines viz; control, M2 50Gy, and M3 reddish brown and white in M3 dark brown line. While M3 brown and gray seeds produced four flower colors: dark purple, purple, light purple, and white. Whereas, M3 grayish brown seeds produced three flower colors: dark purple, purple, and white. Furthermore, γ-radiation induced 15 new seed coat colors in M4 progeny. Molecular characterization showed different fingerprints correlated with the segregated morphological traits and seed coat colors. Both the PCR fingerprints and the dendrograms produced by cluster analysis of the selected four RAPD differentiated clearly between the different M3 segregating genotypes of cv Abo Yousuf from M2 and control plants more than the two ISSR primers. Such findings indicated the pleiotropic effects of seed coat color genes and provided helpful information on gene(s) that govern these traits.

Keywords: DNA markers, Gamma radiation, Mutation, *Phaseolus vulgaris*, Seed coat color.

Introduction

The induction of mutations was a proven way of creating genetic variations with better characteristics to improve crop productivity. The use of gamma radiation (γ-radiation), as a mutagen, for inducing genetic variability was crucial for the breeding features of crop plants (Badr et al., 2014; Ma et al., 2022). Gamma radiation induces genomic instability in cells, such as: single nucleotide mutations, an increase or decrease in genomic copy number, gene amplification, rearrangement, and/or deletion (Morgan, 2003). Mutations induced by γ-radiation are mostly recessive and cannot be selected for breeding before the second generation, where the mutants` homozygous lines might be achieved (Singh, 2005). High doses of γ-radiation might be detrimental as a result of reducing: germination, growth rate, vigor, pollen, and ovule fertility as well as yield (Singh, 2005; El-Azab et al., 2018). On the other hand, at low doses, γ-radiation might help improve crop plants' specific trait(s) (Soliman et al., 2020; Atteh & Adeyeye, 2022).

In *Phaseolus vulgaris*, a correlation between seed coat color and hypocotyl color of M2 seedlings was observed after exposure to γ-radiation. The black seed coat varieties produced red color in hypocotyls, cotyledonary leaves, and leaf veins, while the white seed varieties had a green color in the same organs (Moh, 1971). Okonkwo & Clayberg (1984) proposed a new locus, *Prp* (purple pod), that had five alleles (*Prp*

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prp^{sh}, *prp*^{sh2}, *prp*^{st,} and *prp*) affecting anthocyanin pigmentation of corolla and pods in *Ph. Vulgaris*. Also, they observed an interaction between these alleles with genes Gri, V, and Ro, which also affects the anthocyanin pigmentation of corolla and pods. In addition, a mutation was detected in M2 plants of *Ph. vulgaris* radiated with 20 and 25 KR of γ-radiation affecting flower and seed coat colors (Mahamune & Kothekar, 2011).

Previously, Badr et al. (2014) reported M2 mutant black seeds after the Egyptian cowpea cultivar (Kaha 1) was exposed to a low dose of γ-radiation. Later on, Gaafar et al. (2016) investigated the segregation of seed coat color, the weight of 100 seeds, and the seed eye pattern of this mutant, where they recorded four seed coat colors in the M3 plants ranging from cream to reddish brown and three different eye patterns.

The use of new methods that use high throughput sequencing and new techniques enabled the discovery of hundreds of genetic markers through several genomes that facilitate the study of essential traits (Davey et al., 2011). Genetic markers greatly value plant breeders in isolating, identifying, and evaluating markers-linked genes affecting specific traits (Hasan et al., 2021). RAPD and ISSR markers were used to evaluate the genetic polymorphism between mutant genotypes of many plant species, particularly legumes, including soybean, rice bean, common bean, and cowpea (Souframanien et al., 2002; Muthusamy et al., 2008; Badr et al., 2014; Soliman et al., 2020; Abdalla et al., 2021; El-Badan et al., 2022). McClean et al. (2002) assigned RAPD markers to three genes associated with seed coat color mutations in common beans, and Atak et al. (2004) reported induced mutations in soybean plastid with γ-radiation using RAPD fingerprinting analysis. Genetic markers were converted into easily scorable sequence-tagged site (STS) markers. These markers were further placed onto a common genetic map shared by the *Phaseolus* scientific media and established a wide distribution of the genes throughout the common bean genome. In many crops, γ-radiation increases seeds' protein and amino acids (Yasmin & Chandran, 2022). Protein markers were generated using electrophoresis, taking advantage of the migration properties of proteins and enzymes, and then revealed by specific histochemical stains (Yasmin et al., 2022).

This investigation aims to study the phenotypic and genotypic mutations by γ-radiation low doses on *Ph. vulgaris* cv Abo Yousuf and the segregation of mutant traits like colors of seed coats, hypocotyls, leaf veins, and flower petals in M3 and M4 generations. The genetic diversity of the segregating lines revealed by the two molecular RAPD, ISSR, and protein fingerprinting is also investigated.

Materials and Methods

Seeds irradiation, cultivation, and breeding of M3 and M4 progenies

Dry seeds of *Ph. vulgaris* cv Abo Yousuf was kindly provided by the Agriculture Research Center, Giza, Egypt. The seeds were exposed to γ-radiation at the Atomic Energy Center, Nasr City, Cairo, Egypt using cobalt 60 as a source in the Indian Gamma Cell (Ge 4000 A) at a dose rate of 2.840kgy/h. The three applied doses were 50, 100, and 300Gray (Gy), while seeds used as controls were not exposed to irradiation. Gamma irradiated and control seeds were grown to maturity in an open field experiment in clay loamy soil in the Agriculture College farm at Shibin Elkom city for two successive generations.

The two applied doses, 100 and 300 Gy, had a lethal effect on cv Abo Yousuf seeds. The impact of 50Gy of γ-radiation was evaluated previously in M1 and M2 plants where the harvested M2 seeds showed five different seed coat colors: brown, reddish brown, dark brown, gray, and grayish brown (El-Lithy et al., 2016). In this study, these seeds were further cultivated separately to investigate mutation segregation in the M3 and M4 plants with control and M2 progeny from seeds exposed to 50Gy of γ-radiation. Five seeds from each line were grown in three pots (30cm) in sandy clay soils (1:1 v/v), and the following measurements were recorded: days to germination and flowering, the shape of the first foliage leaf apex, color of hypocotyls, leaves, panicles, flower petals, and pods. After pods ripened, M4 seeds were harvested, classified, and quantified based on their seed coat color. The number of each seed coat color/ the total number of seeds x 100 was applied to obtain the percentage. At the same time, the seeds' weight percentage was obtained by dividing each seed coat color's weight/ its number x 100 (Table 1).

TABLE 1. Seeds number, weight (gm), and percentage of the five M3 seed coat colors of cv Abo Yousuf following parent's seeds exposure to 50Gy of γ-radiation

Seed coat color	Seeds number	Seeds weight (gm)	Seeds number %	Seeds weight %
Reddish brown	819	251.76	64.74	30.74
Dark brown	249	72.76	19.68	29.22
Brown	151	41.24	11.94	27.31
Grayish brown	24	6.13	1.90	25.54
Gray	22	5.09	1.74	23.14

RAPD and ISSR fingerprinting of M3 plants

For DNA extraction, the three seed coat lines viz; M3 brown, M3 gray, or M3 grayish brown gave seedling leaves either with green or purple veins as well as green or purple hypocotyls. Therefore, DNA was extracted separately/ seedlings from these three segregated lines. On the other hand, the seedling leaves and the hypocotyls of the: control, M2 50Gy, M3 reddish brown, and M3 dark brown seed coat lines were all green. Thus the whole seeding leaves were used/for each line. DNA was extracted using Stewart & Via (1993) protocol from young leaves collected in 1.5mL Eppendorf tubes, immediately frozen in liquid nitrogen. DNA from each line was stored at -80ºC for further use. The concentration of DNA was estimated spectrophotometrically at 260 and 280nm, respectively.

Four RAPD and two ISSR primers (Table 2) were selected to reveal polymorphisms following seeds' exposure to γ-radiation. For both RAPD and ISSR primers, the PCR reaction final volume was 20µl containing: 10µL master mix (One PCR™, Gene Direx); 0.6µL primer (100pmol/ µl) (Metabion International AG, Germany); 1µL DNA 50ng/µL and 8.4µL mQ water. The PCR amplification protocols for both RAPD and ISSR primers were done as described by Muthusamy et al. (2008). The amplified fragments were separated on a 1.5% agarose gel (Bio-Rad), at 85 V, for 2h. GeneRuler™ 1Kb Plus DNA Ladder (Fermentas) was a molecular marker. Gels were stained with 3µL/100ml ethidium bromide, and the gel photos were analyzed using UVI soft UVI bandmap Windows Application V11.11.

Protein analysis of M3 plants

Total proteins were extracted according to Fido et al. (2004) and were separated in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) using a mini-gel electrophoresis unit (BioRad, USA), containing 1.25X buffer. The isolated proteins' molecular weight was estimated using a standard molecular weight marker (11-245kDa; Sigma, USA). The protein bands were stained with Coomassie brilliant blue G-250 (Sigma, USA), and the gel was photographed while wet. The gel photos were analyzed by using UVIsoft UVIbandmap Windows Application V11.11.

TABLE 2. Names, nucleotide sequences and annealing temperatures of four RAPD and two ISSR primers used to reveal DNA fingerprinting of M3 lines of cv Abo Yousuf

	Primers	Primer sequence 5`-3´	Annealing temperature (˚C)
		RAPD primers	
1	OPH-03	AGACGTCCAC	35
2	OPW-17	GTCCTGGGTT	35
3	OPC-16	CACACTCCAG	40
4	OPD-12	CACCGTATCC	40
		ISSR primers	
1	A	(GACA)3RT	42
2	D	(ACTG)3RG	42

R= (A, G)

The RAPD and ISSR fingerprinting bands were scored as (1) for presence and (0) for absence in binary matrices for data analysis. The number of unique and polymorphic bands and the percentage of polymorphic bands for each primer and each line were calculated and used to estimate the genetic diversity among the control (C), the M2 50Gy, and the five M3 lines of cv Abo Yousuf following parent's seeds exposure to 50Gy of γ-radiation. For genetic distance estimation, the Community Analysis Package-5 (CAP) (Seaby & Henderson, 2007) was used to construct the average linkage distance tree based on the hierarchical grouping function (Ward, 1963), while the PAST software Version 3.22 based on the paleontological statistics software developed by (Hammer et al., 2001) was used to construct a similarity tree based on the Dice's similarity coefficient. The cluster analysis used the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The principal components analysis (PCA) was applied to assign the variables to genotypes and illustrates the distance of the genotypes in a PCA scatter diagram.

Results

Segregation of growth-related traits of cv Abo Yousuf M3 plants

Four new seed coat colors and the original reddish-brown color appeared in M3 seeds of cv Abo Yousuf following the parent's seeds' exposure to 50Gy. The reddish-brown seed coat color (Plate 1, 1) was scored in 819 out of 1265 seeds with a total weight of 251.76gm and 30.74% (Table 1). The dark brown color (Plate 1, 2) was represented by 249 seeds with a total weight of 72.76gm with 29.22%. The brown color (Plate 1, 3) was scored in 151 seeds with a total weight of 41.24 gm with a percentage of 27.31. On the other hand, the grayish brown color (Plate 1, 4) was scored in 24 seeds with a total weight of 6.13 gm with 25.54%. The gray color (Plate 1, 5) was recorded in the lowest number of seeds (22 seeds), with a total weight of 5.09gm with 23.14% (Table 1).

The M3 plants showed phenotypic segregation among the five planted lines for several morphological and growth traits (Plate 2). These include the segregation of purple and green hypocotyls (A and B), green and purple lower venation of the first foliage leaf (B and C), purple flowers (D), purple pods (E), and emarginated leaf apex (F).

The purple color of hypocotyls, lower venation of the first foliage leaf, panicles, and pods appeared in three lines, brown, gray, and grayish brown seed coat M3 plants, with segregating percentages being 53%, 53%, and 60%, respectively (Table 3). The flower petals were light purple in three lines C, M2 50Gy, and M3 reddish brown, while white in M3 dark brown. M3 gray and M3 brown seed coat plants showed four segregating flower colors (dark purple, purple, light purple, and white) with different percentages for each. M3 grayish brown plants had three flower colors: dark purple, purple, and white. Regarding the first foliage leaf apex, 54% of M3 reddish brown plants were emarginate, 20% had one emarginate and one normal leaf, and 26% had two normal leaves. At the same time, the control plants and the other lines showed 100% normal leaves (Table 3).

Plate 1. Five M3 seed coat colors of cv Abo Yousuf following parent's seeds exposure to 50Gy, where 1: reddish brown; 2: dark brown; 3: brown; 4: grayish brown and 5: gray

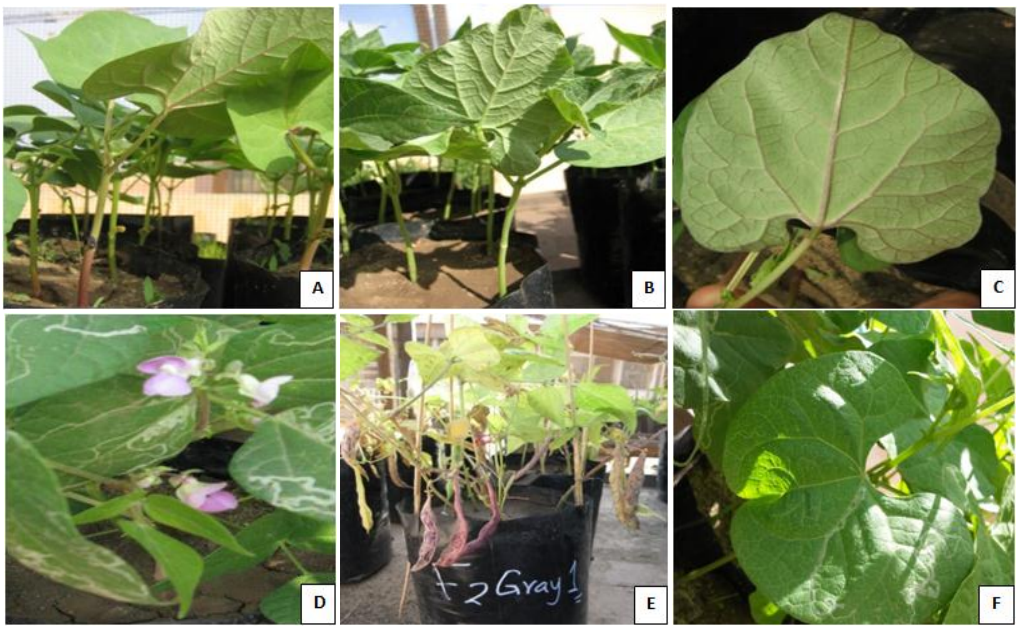

Plate 2. Variations in the morphological traits of M3 plants of cv Abo Yousuf following parent's seeds exposure to 50Gy of γ-radiation. Where A: purple hypocotyl; B: green hypocotyls and green lower venation of the first foliage leaf; C: purple lower venation of the first foliage leaf; D: purple flower; E: purple pods and F: emarginated leaf apex.

TABLE 3. Variation in morphological traits color in the control line (C), M2 treated with 50Gy, and five M3 lines of *Ph. vulgaris* cv Abo Yousuf grown from M2 seeds following parent's seeds exposure to 50Gy of γ-radiation. The traits include the colors of: hypocotyls, lower venations of the first foliage leaf, panicles, flower petals, pods, and shape of the first foliage leaf apex

Morphological traits	C	M2 50Gy	M3 Reddish brown	M3 Dark brown	M3 Gray	M3 Grayish brown	M3 Brown
Hypocotyls color	G	G	G	G	53% P 47%G	60% P 40% G	53% P 47% G
1st foliage leaf venation color	G	G	G	G	53% P 47% G	60% P 40% G	53% P 47% G
Panicle color	G	G	G	G	53% P 47%G	60% P 40% G	53% P 47% G
Flower petals color	LP	LP	LP	W	31% DP 15% P 31% LP 23% W	47% DP 13% P 40% W	13% DP 27% P 40% LP 20% W
Pods color	G	G	G	G	53% P 47%G	60% P: 40% G	53% P 47% G
1st foliage leaf apex	100% N	100% N	26%N: 20% N&E 54% E	100% N	100% N	100% N	100% N

G: green, **P**: purple, **LP**: light purple, **DP**: dark purple, **W**: white, **N**: normal and **E**: emarginate.

The seeds of the M3 dark brown line were the first to germinate after 4.2±0.41 days, followed by the seeds of the M3 grayish brown line, which germinated after 4.2±0.85 days (Fig. 1). The seeds of the control line showed delayed germination (5.07±0.59 days) compared to the M3 lines. On the other hand, the M2 50Gy and the M3 dark brown seed coat color lines were the first to flower after 35±2.86 and 35±3.16 days, respectively. Both the control and the M3 reddish brown line plants flowered after 38.86±1.23 and 38.47±3.38 days, respectively (Fig. 1).

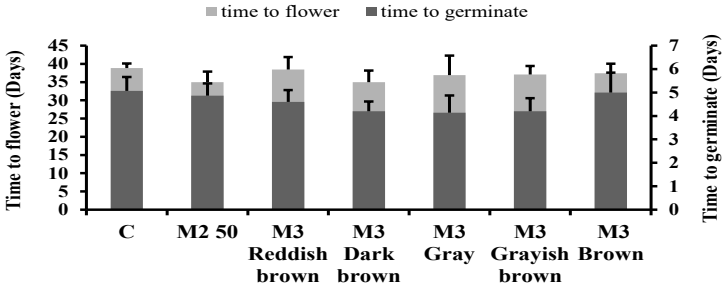

Fig. 1. Time of germination and flowering of the control (C), the M2 50Gy and five M3 lines of cv Abo Yousuf following parent's seeds exposure to 50 Gy of γ-radiation

The harvested M4 seeds from the M3 plants revealed 15 new seed coat colors (Plate 3 and Table 4). When reexamined after eight months of storage at 4ºC, such colors were the same. The parent M3 reddish brown seed coat color produced two new seed coat colors in the M4 seeds: 85% cream (Plate 3A) and 15% cream with dark mosaic violet venation (Plate 3M). The M3 dark brown seed coat line segregated into five seed coat colors: 50% white (Plate 3C), 21.4% cream with mosaic violet venation (Plate 3N), 14.3% dark brown (Plate 3K), and 7.1% for both dark slate gray (Plate 3L) and cream (Plate 3A and Table 3). The M3 gray seed coat color was segregated into seven classes in the M4 progeny. The most common color was cadet blue, 30.7% (Plate 3I), followed by white (23.1%). Both the dark blue (Plate 3D) and the cream with mosaic violet venation (Plate 3N) were represented by 15.3% each. The lowest seed coat colors were scored as dark tan (Plate 3B) or corn silk (Plate 3J), or sienna (Plate 3E), where each color was represented by 7.6% (Table 4).

Plate 3. The 15 new seed coat colors segregated in M4 seeds of cv Abo Yousuf following parent's seeds exposure to 50Gy of γ-radiation, where A: Cream, B: Dark tan, C: White, D: Dark blue, E: Sienna, F: Dull black, G: Glossy black, H: Burly Wood, I: Cadet Blue, J: Corn silk, K: Dark brown, L: Dark Slate Gray, M: Cream with dark mosaic violet venation, N: Cream with mosaic violet venation, O: Pero

The M3 grayish brown seed coat color was segregated into eight classes in the M4 progeny. At the same time, dark tan and cream were the most common, with 26.7% each. The white seed coat color scored 13.3%, while the other colors (glossy black, burly wood, sienna, dark blue, and dull black) scored 6.7% each (Plate 3 and Table 3). The M3 brown seeds produced seven classes of seed coat colors in the progeny of the M4 seeds, where sienna and cadet blue represented 20% each. The other four colors (white, cream, cream with mosaic violet venation, and Pero) scored 13.3% for each. The dull black color was the lowest representing only 6.7% of the progeny of the M4 seeds (Plate 3 and Table 4).

DNA fingerprinting of M3 lines of cv Abo Yousuf

Four RAPD primers viz., OPH-03, OPW-17, OPD-12, and OPC-16 were used to analyze the DNA fingerprinting of the eight M3 segregating lines and the control and the M2 50 of cv Abo Yousuf. The RAPD profile of the OPH-03 primer revealed PCR products ranging from 398 bp to 2211 bp. Five monomorphic bands (normal arrows) at 2117, 1030, 880, 688, and 400bp could be detected for all lines except line brown-green at 880bp. A polymorphic band (notched arrow) at 508bp was present only in two lines; gray-purple, and gray-green (Suppl. Fig. 1a). The RAPD fingerprinting (Suppl. Fig. 2a) could not discriminate between the genotypes of M2 50 and control lines or between M3 gray-green and M3 gray-purple. The control line and M2 50 were clustered far from the other genotypes. The original seed coat color (M3 reddish brown)

was grouped with low homology (60%) with the M3 dark brown genotype (group I). The two segregating genotypes, M3 brown-purple and M3 brown-green, were grouped with moderate homology being 88%. The two segregating genotypes (M3 grayish brown purple and M3 grayish brown-green) were linked separately to other genotypes at 80% similarity and then clustered at 69% (group II).

TABLE 4. The M3 and M4 seed coat colors and the percentage of their segregation in M4 seeds of cv Abo Yousuf following parent's seeds exposure to 50Gy of γ-radiation

M3 seed coat colors	M4 seed coat colors	M4 percentage
Reddish brown	Cream	85.0%
	Cream with dark mosaic violet venation	15.0%
Dark brown	White	50.0%
	Cream with mosaic violet venation	21.4%
	Dark brown	14.3%
	Dark Slate Gray	7.1%
	Cream	7.1%
Gray	Cadet Blue	30.7%
	White	23.1%
	Dark blue	15.3%
	Cream with mosaic violet venation	15.3%
	Dark tan	7.6%
	Corn silk	7.6%
	Sienna	7.6%
Grayish brown	Dark tan	26.7%
	Cream	26.7%
	White	13.3%
	Glossy black	6.7%
	Burly Wood	6.7%
	Sienna	6.7%
	Dark blue	6.7%
	Dull black	6.7%
Brown	Sienna	20.0%
	Cadet Blue	20.0%
	White	13.3%
	Cream	13.3%
	Cream with mosaic violet venation	13.3%
	Pero	13.3%
	Dull black	6.7%

The RAPD profile of OPW-17 primer revealed PCR products ranging from 207 bp to 756 bp. All lines could see one monomorphic band (normal arrow) at 606bp. Three polymorphic bands (notched arrows) at 745, 470, and 207bp were present only in four lines; dark brown, reddish brown, M2 50Gy, and control, except line M2 50Gy at 470bp. Also, one polymorphic band (notched arrow) at 484bp was specific only in two lines; gray-purple, and gray-green. While the two bands at 357 and 291bp (notched arrows) were characteristic for six genotypes; brown purple, brown green, grayish brown purple, grayish brown green, gray purple, and gray-green (Suppl. Fig. 1 b). The dendrogram based on the analysis of this profile (Suppl. Fig. 2 b) revealed 100% similarity between the segregating lines M3 brown-purple, M3 brown-green, M3 grayish brown purple, and M3 grayish brown-green. Both M3 gray purple and M3 gray-green, as well as M3 dark brown and M3 reddish brown, were clustered at 100% similarity. On the other hand, M3 gray lines (purple and green) clustered with the M3 grayish brown (purple and green) and M3 brown lines (purple and green) at 57% similarity (group II). The dendrogram showed that M3 reddish brown and M3 dark brown were closely clustered to the control and the M2 50 lines (group I).

The RAPD profile of the OPC-16 primer revealed PCR products ranging from 386bp to 3827bp. The two bands, 3827 and 386bp, were characteristic of the reddish brown, M2 50Gy, and control lines, while they were absent in the others. A specific band at 2893bp was present only in the reddish-brown genotype. The 2474bp band was characteristic of the brown-purple and grayish brown green samples, while it was absent in the others. The 1628bp was characteristic of five genotypes; brown green, grayish brown purple, grayish brown green, gray purple, and gray-green. The 1468bp band was present only in the brown purple and dark brown samples. The 716bp band was absent in the brown purple, and gray-green lines while present in the others. Also, the 459bp band was absent in the reddish brown, M2 50Gy, and control lines while present in the others (Suppl. Fig. 1c). The dendrogram based on this profile showed genetic diversity between the examined genotypes (Suppl. Fig. 2c). At 75% similarity, the lines M3 brown-green, M3 grayish-brown purple, and M3 gray-purple were grouped. This group was linked at 66% similarity to M3 grayish-brown-green and M3 gray-green (group II). The dendrogram showed that M2 50 was linked to the control at 66% similarity and then to M3 reddish brown at 28% similarity (group I), where these three lines were separated from the other genotypes.

The RAPD profile of the OPD-12 primers revealed PCR products ranging from 488 bp to 978 bp. The three lines, grayish brown-green, gray-green, and M2 50Gy, did not give any PCR products. A 978bp band was characteristic of the brown-green, reddish brown, and control. The 771bp was present in the grayish-brown purple, gray purple, and dark brown lines. While the 488bp was present in all genotypes (Suppl. Fig. 1 d). The dendrogram based on this profile showed two groups; the first group comprises the five genotypes: M3 dark brown and M3 gray-purple (with 100% similarity), M3 reddish brown, M3 grayish brown purple, and M3 brown green. The second group includes the control line and the M3 brown-purple, which clustered at 66% similarity (Suppl. Fig. 2d).

The ISSR analysis of the ten lines was conducted using two primers viz., A and D. The ISSR profile of (A) primer revealed PCR products ranging from 142 to 1500bp. A 1500bp band was absent only in reddish brown, while it was present in the others. The 659bp band was characteristic of the M2 50Gy and the control lines, while it was absent in the others. The 459 bp band was characteristic of brown purple, brown green, grayish brown green, and reddish brown lines while disappearing in the others (Suppl. Fig. 3a). The four lines, M3 brown, purple, M3 brown-green, M3 grayish brown purple, and M3 grayish brown-green, clustered as one group (group II) with similarity ranging from 66 to 100%. The other genotypes clustered in group I. In the latter group, the M3 gray purple and the M3 gray green were clustered with 100% similarity, and the control line with the M2 50 line. Also, in the latter group, the control and the M2 50 line were linked to the M3 dark brown genotype with 60% similarity (Suppl. Fig. 4a).

The ISSR profile of (D) primer revealed PCR products ranged from 607 to 4000bp. Two bands, 4000bp and 607bp, were absent only in control, while it was present in the others. A monomorphic band at 1631 could be detected for all genotypes (Suppl. Fig. 3b). The dendrogram showed that lines M2 50, M3 reddish brown, M3 dark brown, M3 gray-green, M3 gray purple, M3 grayish brown green and M3 grayish brown-purple were clustered

together at 100% similarity (group I), as well as M3 brown purple and M3 brown-green (group II). The two groups were linked to each other at 66% similarity. The control line was assigned far away from the M3 genotypes (Suppl. Fig. 4b).

Figure 2 illustrates the genetic diversity of the eight M3 seed coat segregating genotypes, in addition to control and M2 50Gy of cv Abo Yousuf as a cluster tree (A) and a PCA scatters diagram (B). In the cluster tree, one group, comprised of the control, M2 50Gy, and the M3 reddish brown (M3 RB) lines, were distinguished from another major group. The latter group is divided into two groups, one including the three lines M3 Gray Green (M3 GG), M3 Gray Purple (M3 GP), and M3 Dark Brown (M3 DB). The other group includes the M3 G. brown-purple (M3 GBP), the M3 G. brown green (M3 GBG), the M3 brown purple (M3 BP), and the M3 brown green (M3 BG).

In the PCA scatter diagram (Fig. 2B), the control, the M2 50Gy, and the M3 RB are clearly grouped close to each other and distinguished from the other seven lines. These lines were separated as two groupings resembling the groups distinguished in the cluster analysis, one comprising three lines, M3 GG, M3 GP, and M3 DB. While the other one includes M3 GBP, M3 GBG, M3 BP and M3 BG lines.

Protein SDS-PAGE analysis of M3 generation of cv Abo Yousuf

Protein analysis using SDS-PAGE revealed 99% polymorphism between two groups; group I had four genotypes named brown-purple, brown-green, grayish-brown-purple, and grayish-brown-green. While group Π was divided into two clusters; one comprises the gray-green and gray purple with 100% similarity, and the dark brown genotype, distinguished at 54% polymorphism. The other cluster includes the control, M2 50, and the M3 reddish brown genotypes (Fig. 3).

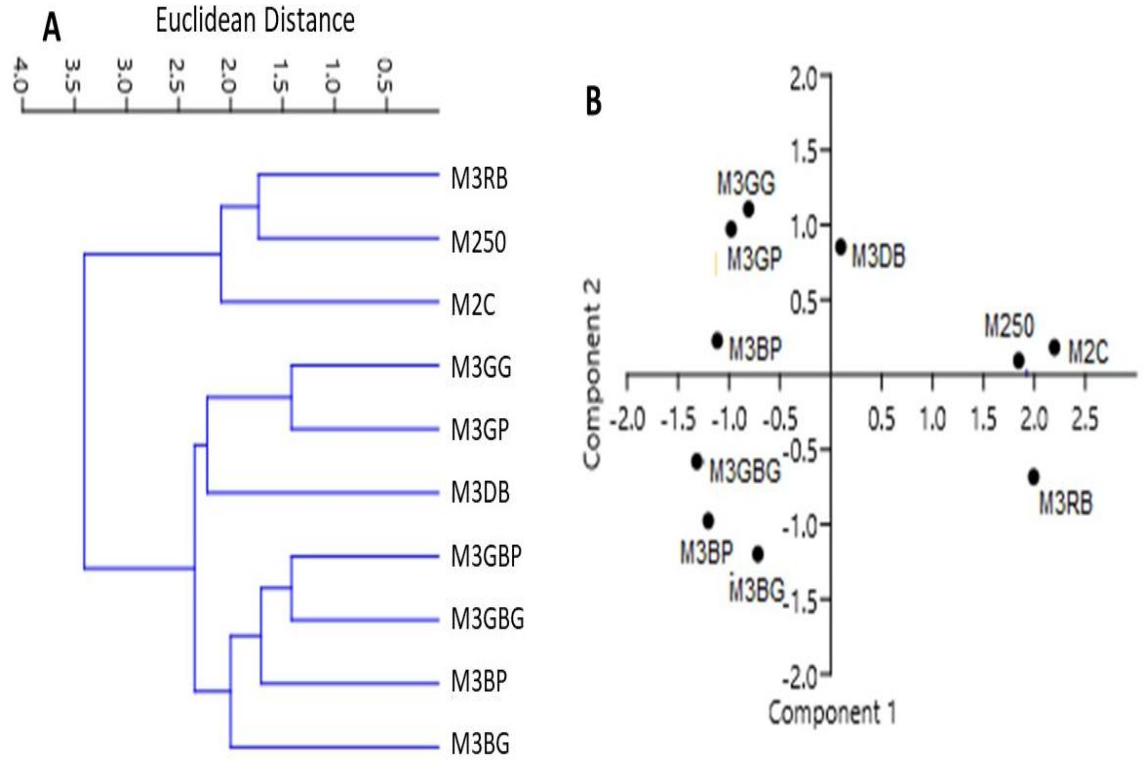

Fig. 2. Dendrogram illustrates the differentiation of eight M3 seed coat segregating genotypes, in addition, to control and M2 50Gy of cv Abo Yousuf as a cluster tree (A) and a PCA scatters diagram (B) using the Past software based on 4 RAPD (OPH-03, OPW-17, OPC-16 and OPD-12) and 2 ISSR (A and D) primers fingerprinting polymorphisms. M3RB = M3 reddish brown, M250 = M2 50Gy, M2C = M2 Control, M3GG = M3 gray green, M3GP = M3 gray purple, M3DP = M3 dark brown, M3GBP = M3 gray brown purple, M3GBG = M3 gray brown green, M3BP = M3 brown purple, and M3BG = M3 brown green

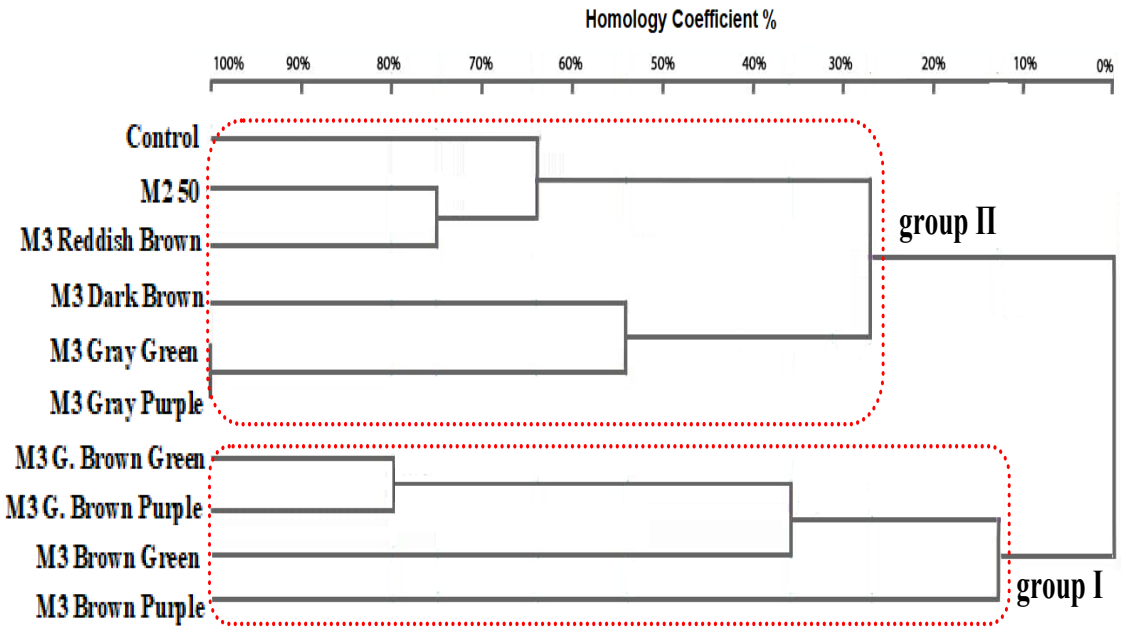

Fig. 3. Dendrogram illustrates the differentiation of eight M3 seed coat segregating genotypes, in addition, to control and M2 50Gy of cv Abo Yousuf based on protein fingerprinting polymorphisms. G= grayish

Discussion

Exposure to γ-radiation produces morphological, physiological, and biochemical mutants (Yasmin & Chandran, 2022). Several reports, including Badr et al. (2014), on the M2 plants of cowpea; El-Azab et al. (2018), on soybean cultivars; Abdoun et al. (2022) on chickpea varieties and Labeeb et al. (2022) on pea (*Pisum sativum*) concluded that high doses of γ-radiation were usually inhibitory whereas, low doses were stimulatory of plant growth and development as well as yield. Treatments of common bean seeds with 100 and 300Gy of γ-rays were lethal (El-Lithy et al., 2016). In this work, the M3 generation of common bean of cv Abo Yousuf showed four new seed coat colors, and their segregation in the M4 generation which produced 15 new seed coat colors, is reported.

The results in this study agree with Moh (1971), who was the first to report changes in seed-coat colors of *Ph. vulgaris* after γ-radiation in the M3 seeds. There is a correlation between the seed-coat colors and the hypocotyl color of the seedling. The black-seeded varieties produced a red color in the hypocotyls, cotyledon, and leaf veins, whereas the white-seeded varieties produced a green color in the same organs (Moh, 1971). Also, Mahamune & Kothekar (2011) detected mutations affecting flower color and seed coat color in M2 plants of *Ph. vulgaris* radiated with 20 and 25KR of γ-rays. These colors are due to the expression of new segregating alleles from the gene(s) responsible for seed coat colors. Prakken (1974) and Ergun et al. (2001) reported more information on genes controlling seed coat colors in common beans. The gene P is essential for color development, but the gene T is necessary for a uniform-colored seed coat. The gene locus for seed color C, is a complex locus with different alleles and for constant mottling; D is the hilum ring factor; J is the shine factor; G is the yellow-brown factor; B is the gray-greenish brown factor; V is the violet factor; and Rk, for reddish brown color. Gaafar et al. (2016) recorded that black cowpea seeds of M2 generation, after exposure to 50 Gy of γ-radiation, were segregated into various categories depending on seed coat color in the M3 generation. Also, Gnanamurthy et al. (2012) recorded mutations for seed and pod colors in the M3 generation of cowpea by γ-radiation.

In this study, the purple color appeared in the hypocotyls, lower venation of the first foliage leaf, panicle, flower petals, and pods of the M3 plants. Previous results indicated that a new locus, *Prp* (purple pod), had five alleles affecting anthocyanin

pigmentation for both corolla and pods (Okonkwo & Clayberg, 1984). The allele *Prp* produces the dark-purple corolla and is dominant over the other four alleles that determine the light-purple corolla. In the absence of *Ro*, *Prp* is responsible for medium-purple pods when homozygous and light-purple pods combine with the other alleles. The alleles prp^{sh}, and prp^{sh2} give green pods shaded with purple and are co-dominant with the prp^{st} allele, which causes green pods to be striped with purple. These four alleles are dominant for pod color over *prp*, an allele causing green pods when homozygous. These alleles had interactions with the genes *Gri*, *V*, and *Ro*, which also affect the anthocyanin pigmentation of corolla and pod. Horn et al. (2016) scored segregation in flower color (white and purple) at the M2 cowpea plants after its radiation with γ-rays. Also, they scored variations in seed coat colors.

Regarding growth criteria such as time to germinate and flower, the M3 lines after γ-radiation were slightly earlier than their controls. On the other hand, Atteh & Adeyeye (2022), when investigating another growth criterion, i.e., the germination percentage, reported that the dose of γ-radiation had no significant effect when applied to the three legumes: broad beans, mung beans, and peas seedlings. At the same time, flowering time was delayed in irradiated cowpea M1 plants than the corresponding control, while M2 and M3 generations flowered earlier than the control at 400Gy γ-radiation and showed late flowering at 800Gy compared respectively to control and other doses. Seed yield increased at 200Gy in both generations of M2 and M3 (Vanmathi et al., 2021).

The PCR fingerprints of selected four RAPD and two ISSR primers discriminate between the new M3 genotypes of cv Abo Yousuf through specific bands characteristic to certain segregating genes. Also, the dendrograms produced by the cluster analysis differentiated between the different M3 segregating genotypes that reflect the existing polymorphism. The cluster and PCA analyses differentiated between the M3, the M2 and the control plants. The variation between the different M3 genotypes might result from new gene mutations in the genome due to nucleotide sequence changes in the DNA. Gonzàlez et al. (2005) assessed the ability of ISSRs as molecular markers to identify genetic diversity both within and among populations of common beans, including one wild and four domesticated populations. Galvan et al. (2003) used 23 ISSR and 16 RAPD primers to assess genetic diversity and determine the relationships among 13 common bean cultivars; 40% of the ISSR primers were polymorphic, while only 25% of the RAPD primers were polymorphic. The effects of ISSR fingerprinting might be connected to structural rearrangements in DNA caused by different types of DNA damage (Mejri et al., 2012).

These changes are also due to variations in the protein patterns, which may result from the denaturation of protein or protein deamination (Abu et al., 2005). Changes in protein synthesis following γ-radiation treatments may be due to changes in the efficiency of mRNA translation or the regulation of RNA transcription transport and stability, as Mohamed (2011) reported. Berber & Yasar (2011) could differentiate between 28 cultivars of *Ph. vulgaris* based on their protein profiles. Badr et al. (2014) used the SDS-PAGE technique to show the variation in the banding pattern of seed protein polypeptides for the M2 generation of cowpea following seeds' exposure to the applied doses of γ-radiation. Khursheed & Khan (2017) showed a considerable increase in mutants` proteins compared to the control using SDS-PAGE when two cultivars of *V. faba* L were radiated with γ-rays. They stated that the considerable increase in proteins might be due to the stimulation of RNA.

Conclusion

The current study produced new genotypes in the M3 generation of *Phaseolus vulgaris* cv. Abo Yousuf, due to exposure to γ-radiation, has been differentiated by seed coat color in the M3 to brown, reddish-brown, dark brown, grayish brown, and gray. While, the M4 generation showed 15 new seed coat colors viz; Dark tan, Cream, White, Glossy black, Burly Wood, Sienna, Dark blue, Dull black, Cream with mosaic violet venation, Cadet Blue, Pero, Corn silk, Dark tan, Dark brown, and Dark Slate Gray. Colors of hypocotyls, panicles, leaf venations, flower petals, and pods were correlated with seed coat color mutants. Control, M2 50Gy, M3 reddish brown, and M3 dark brown seed coat lines were green with light purple flowers. While M3 gray, M3 grayish brown, and M3 brown seed coat lines are segregated into purple and green colors with either dark purple, purple,

light purple, or white flowers. The γ-radiation, at low doses, accelerates both the germination and the flowering time of three genotypes: M3 dark brown, M3 gray, and M3 grayish brown. RAPD and ISSR primers were able to characterize the genetic variation that exists between the different seed coat lines. The cluster analysis using RAPD primers could discriminate between the different lines more clearly than the ISSR primers.

Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: M. El-Lithy and Abdelfattah Badr have contributed in suggesting and design of the work, interpretation of data and discussion, and final revision of the manuscript. M. El-Lithy and S. Abdelgawad carried out experiments and has performed the practical part and wrote the draft of the manuscript. M. El-Lithy handled both gel images and statistical analysis. All authors are in agreement with the contents of the manuscript.

Ethics approval: Not applicable.

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الطفرات الشكلية والجينية التي تحثها أشعة جاما في نباتات الجيل الثالث والرابع لنبات الفاصوليا(*Phaseolus vulgaris*)

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تعرضت بذور نبات الفاصوليا البنية المحمرة اللون (*Phaseolus vulgaris* L. cv Abo Yousuf) إلى 50Gy من إشعاع جاما و الذي أنتج بذور M2 بألوان غلاف مختلفة. تمت زراعة هذه البذور بشكل منفصل للتحقيق في إنعزال بعض الصفات في أجيال نباتات M3 و M4 مقارنة بالنباتات المرجعية (الأباء) وذرية M2. أظهرت بذور M3 خمسة ألوان من غلاف البذور: بني، بني محمر، بني داكن، بني رمادي، ورمادي. في البذور M3 ، كان اللون البني المحمر هو الأكثر انتشارًا وسجل متوسط وزن 100 بذرة الأعلى ، بينما كان لون البذور الرمادية الأقل ملاحظة وله وزن 100 بذرة أخف بكثير. لوحظ الانعزال في الصفات الوراثية بين hypocotyls الأرجواني والأخضر، والتعرق الخلفي الأرجواني في الورقة الأولى من أوراق النباتات، بالإضافة إلى الزهرة الأرجواني، والقرون الأرجوانية، وقمة الأوراق في نباتات M3. حيث تم فصل اللون الأرجواني إلى ثلاثة خطوط؛ البني والبني الرمادي والرمادي. وكانت الزهرة أرجوانية فاتحة في ثلاثة خطوط وراثية: الأباء، M2 50Gy، و M3 بني محمر، وأبيض في الخط M3 البني الغامق. بينما أنتجت البذور الرمادية M3 و M3 أربعة ألوان للزهور (أرجواني داكن، بنفسجي، بنفسجي فاتح، وأبيض). بينما أنتجت البذور البنية الرمادية M3 ثلاثة ألوان للزهور (أرجواني داكن، بنفسجي، أبيض). أيضًا، تسبب الإشعاع في ظهور 15 لونًا جديدًا لغلاف البذور في ذرية M4. أظهر التوصيف الجزيئي بصمات مختلفة مرتبطة بفصل الصفات المورفولوجية المدروسة وألوان غلاف البذور. أشارت هذه النتائج إلى التأثيرات المتعددة الاتجاهات لجينات لون غلاف البذور وتوفر معلومات مفيدة عن الجين (الجينات) التي تحكم هذه السمات.